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Spectrophotometric analysis of organisation of dipalmitoylphosphatidylcholine bilayers containing the polyene antibiotic amphotericin B

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Abstract

Amphotericin B (AmB) is a polyene antibiotic widely used in the treatment of deep-seated fungal infections. The mode of action of AmB is directly related to the effect of the drug on the lipid phase of biomembranes. In the present work the effect of AmB on the properties of lipid bilayers formed with dipalmitoylphosphatidylcholine (DPPC) and the effect of the lipid phase on the molecular organisation of AmB were studied with application of spectrophotometry in the UV-Vis region. The absorption spectra of AmB in lipid membranes display a complex structure with hypsochromically and bathochromically shifted bands indicative of formation of molecular aggregates of the drug. Formation of molecular aggregates was analysed at different concentrations of the drug in the lipid phase in the range 0.05–5 mol% and at different temperatures in the range 5–55°C. The aggregation level of AmB in the ordered phase of DPPC displayed a minimum corresponding to a concentration of 1 mol% with respect to the lipid. An increase in the aggregation level was observed in the temperature region corresponding to the main phase transition. The structure of molecular aggregates of AmB is analysed on the basis of spectroscopic effects in terms of the exciton splitting model. Analysis of the position of the absorption maximum of AmB in the lipid phase of DPPC in terms of the theory of solvatochromc effects makes it possible to ascribe the refractive indices $n = 1.40$ and $n = 1.49$ to the hydrophobic core of the membrane in the L_α and the P_β' phase respectively. Analysis of the aggregation of AmB in the lipid phase in relation to the physical state of the membrane reveals that the temperature range of the main phase transition of a lipid cluster in the immediate vicinity of AmB depends on its concentration. The termination of the phase transition temperature, as read from the AmB aggregation, varies between 42°C at 1 mol% AmB in DPPC and 49°C at 5 mol% AmB in DPPC. The exciton splitting theory applied to the analysis of the spectroscopic data makes it possible to calculate the diameter of the AmB pore as 2.8 Å in the gel phase and 3.6 Å in the fluid phase of the DPPC membrane, on the assumption that the pore is formed by nine AmB molecules.

Keywords: Amphotericin B; Liposome; Molecular aggregate; Polyene antibiotic; Membrane pore

1. Introduction

Amphotericin B (AmB) is one of the main polyene antibiotics widely used to treat deep-seated fungal infections [1]. The mechanism of biological action

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of AmB is most probably directly related to the ability of the drug to form hydrophilic pores in the hydrophobic membrane core, thus affecting physiological transport of ions [1–4]. One of the possible effects of AmB with respect to the membrane is based on the interaction of the drug with sterols, promoting formation of membrane pores [2,3], and/or by decreasing the concentration of sterols within the lipid phase. The interaction of AmB with lipids has been the subject of numerous studies [2–12] including those carried out with the application of UV-visible spectroscopy [4,6,13,14]. Incorporation of AmB into the hydrophobic core of the membrane results in the formation of molecular aggregates that probably have a form of hydrophilic pores composed of six to nine molecules [2,3,15]. On the other hand, the formation of reversed pores has been postulated in a hydrophilic environment [14]. Rigid molecules of AmB also modify structural and dynamic properties of lipid membranes, as demonstrated by calorimetry [9–11], the spin probe technique [7,8] and ultrasound absorption measurements [10]. The effect of AmB consists in formation of cooperative units of lipid molecules characterised by the main phase transition temperatures being shifted toward higher values. This effect is demonstrated by a decrease in cooperativity of the phase transition. In the present work both the effect of the physical state of the dipalmitoylphosphatidylcholine (DPPC) membrane on formation of AmB aggregates and the effect of AmB on membrane properties are studied based on analysis of electronic absorption spectra. The fact that the spectral properties of AmB itself are applied to probe an effect of the drug on membrane lipids provides a unique possibility of analysing directly the mechanism of lipid–AmB interaction in a bilayer.

2. Materials and methods

DPPC for liposomes and AmB were purchased from Sigma. Unilamellar liposomes were formed with pure lipid (1.5 mg/ml DPPC) or DPPC with 0.05, 0.1, 1, 2 or 5 mol% AmB. A stock solution of DPPC was prepared in ethanol and a stock solution of AmB in 40% 2-propanol. AmB-containing DPPC liposomes were prepared according to the procedure described in detail previously [10]. Briefly, the required amounts of AmB and DPPC were mixed in a glass tube and evaporated under a stream of nitrogen. In order to remove residuals of solvents, a thin film of DPPC–AmB was placed in a vacuum for 2 h. Large multilamellar vesicles were formed by vortexing (for 10 min) a lipid film hydrated in Tricine buffer, 0.1 M, pH 7.6. A liposome suspension was then subjected to sonication for 1 min with a 20 kHz Unipan ultrasonic disintegrator equipped with a titanium probe at a temperature of 45°C, above the main phase transition of DPPC. The diameters of liposomes prepared according to this procedure were in the range 69–130 nm as checked by the method of quasi-elastic light scattering (around 80% between 90 and 110 nm). Following preparation, the liposome suspension was subjected to centrifugation at $15\,000 \times g$ in order to remove AmB aggregates possibly not incorporated into liposomes. No AmB microcrystals were found after the sonication.

Electronic absorption spectra of AmB-containing liposome suspensions were recorded against pure DPPC liposome suspensions with a double-beam UV-Vis Shimadzu spectrophotometer model 160A-PC equipped with a thermostated cuvette holder. Absorption spectra were recorded in the temperature range 5–55°C. The temperature was controlled with a PolyScience thermostated circulator and monitored with the NiCl–NiAl thermocouple placed directly within the sample. Spectral analysis was performed with Grams 32 software from Galactic Industries.

3. Results and discussion

Figs. 1 and 2 present the absorption spectra of DPPC liposomes containing 1 and 5 mol% AmB respectively, along with the Gaussian analysis of the absorption bands. Both spectra contain the same principal absorption bands characteristic of AmB in monomeric and aggregated forms [4,6,11,13–15] but the actual proportion of bands depends on the sample composition. The Gaussian components centred at 408, 384 and 370 nm correspond to the 0-0, 0-1 and 0-2 vibronic transitions of the main electronic absorption band of AmB in the monomeric form whereas the Gaussian bands centred at 350, 331 and 317 nm can be ascribed to the

corresponding excitonic bands of AmB in the oligomeric state. Long-wavelength-shifted absorption bands are also visible in the spectra, which most probably represent the transitions to the low-energy excitonic bands of the aggregated AmB. Clearly, at a first approximation the higher the concentration of AmB, the higher the aggregation level represented by the intensity of hypsochromically shifted absorption bands with respect to the absorption bands characteristic of the monomeric form of the drug. The same effect can be also seen from Fig. 3. Fig. 3 presents the temperature dependences of absorption spectra of AmB-containing liposomes. As can be seen, a distinct transformation of spectral properties of AmB corresponds to the main phase transition temperature of DPPC (~41°C). The level of aggregation of AmB depends on the temperature, but the actual position of the absorption bands of monomeric AmB also appears to be different at temperatures below and above the phase transition. This can be seen from Fig. 4, where the positions of the 0-0 band in the absorption spectra of AmB in liposomes are presented against incubation temperatures. The 0-0 band adopts essentially two different positions at

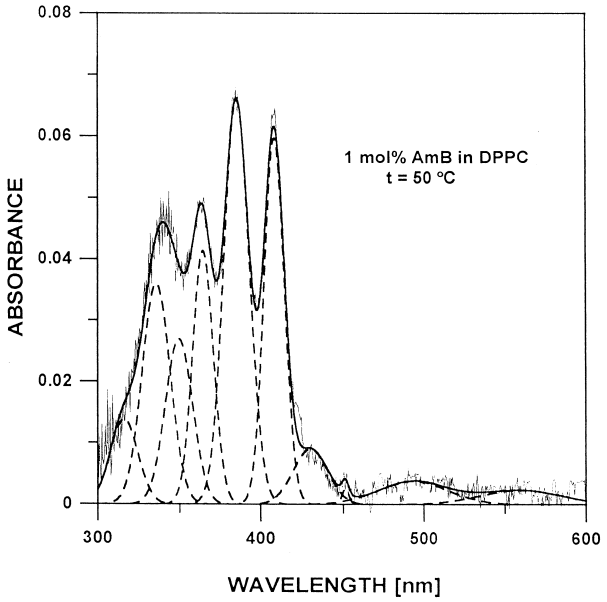

Fig. 1. Absorption spectrum of the suspension of DPPC liposomes containing 1 mol% AmB, recorded at 50°C along with the Gaussian analysis. The original spectrum and the reconstituted spectrum are drawn with a thin and thick solid line, respectively, the Gaussian components with a dashed line.

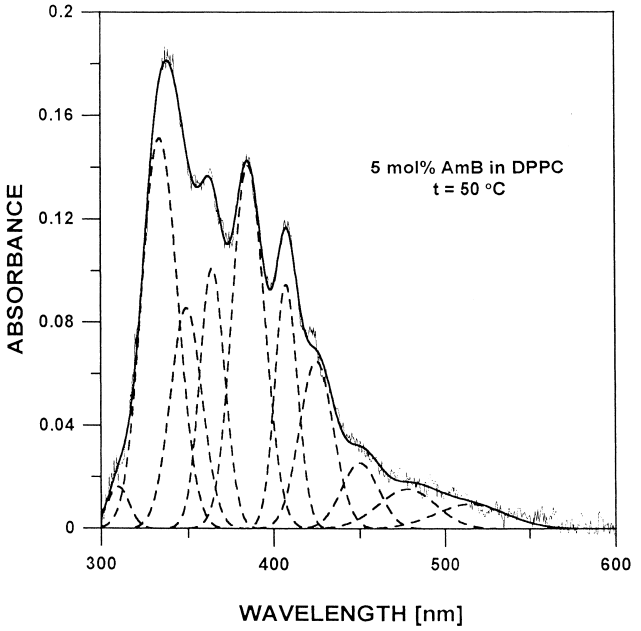

Fig. 2. Absorption spectrum of the suspension of DPPC liposomes containing 5 mol% AmB, recorded at 50°C along with the Gaussian analysis. The original spectrum and the reconstituted spectrum are drawn with a thin and thick solid line, respectively, the Gaussian components with a dashed line.

low AmB concentrations, one at temperatures below and the other at temperatures above the phase transition, but the same band shifts gradually towards higher wavenumbers (lower wavelengths) in the course of the increase in incubation temperature at higher AmB concentrations. Such an effect may be interpreted in terms of the solvatochromism theory and the dependence of transition energy on dielectric properties of a chromophore environment [16]. Especially at low AmB concentrations, below the aggregation threshold in the membrane (0.1 mol% [4]) the drug may be regarded as a probe reporting properties of a hydrophobic core. At higher AmB concentrations the picture appears to be more complex and apparently other phenomena are involved, most probably related to the observed decrease in the cooperativity of the phase transition discussed above. Fig. 5 presents a correlation of the position of the 0-0 absorption band of AmB and the polarisability term, obtained for a series of organic solvents. From this correlation the values of a refractive index of the hydrophobic core of DPPC in the P_β' phase and in the L_α phase can be found as $n = 1.49$ and $n = 1.40$, based on the positions of the absorption maxima of

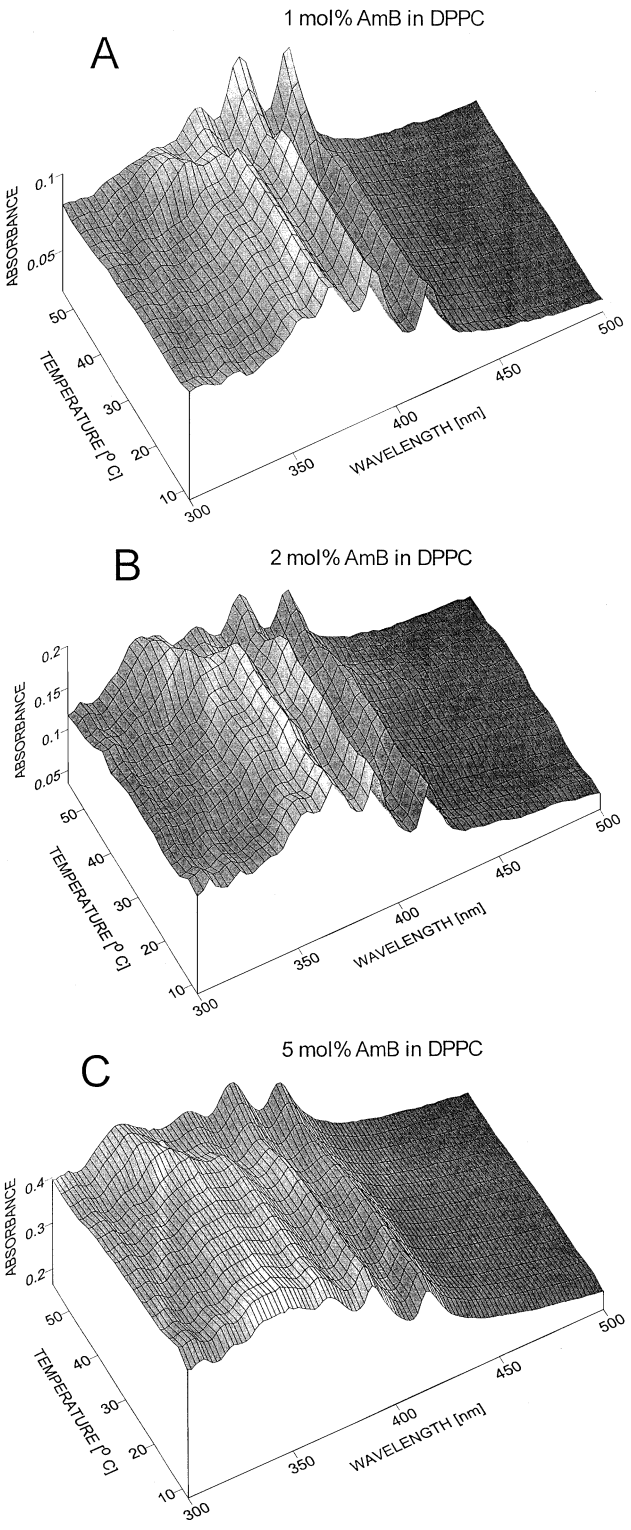

Fig. 3. Temperature dependences of absorption spectra of DPPC liposomes containing AmB at 1 (A), 2 (B) and 5 mol% (C).

AmB in DPPC at 0.05 and 0.1 mol%. These values are very close to the refractive index of the hydrated dioleoyl phosphatidylcholine determined as $n = 1.44$ using the Brewster angle method [17].

Fig. 6 presents the temperature dependences of the ratio of intensity of the aggregation band centred at 334 nm (I_A) and the 0-0 band of a monomer (I_M). Parameter I_A/I_M is directly related to the aggregation level of AmB within the membrane, dependent on the concentration of the drug and the physical state of the membrane, in particular membrane structure and fluidity. As may be seen, the fluidisation of the membranes following the transition to the disordered

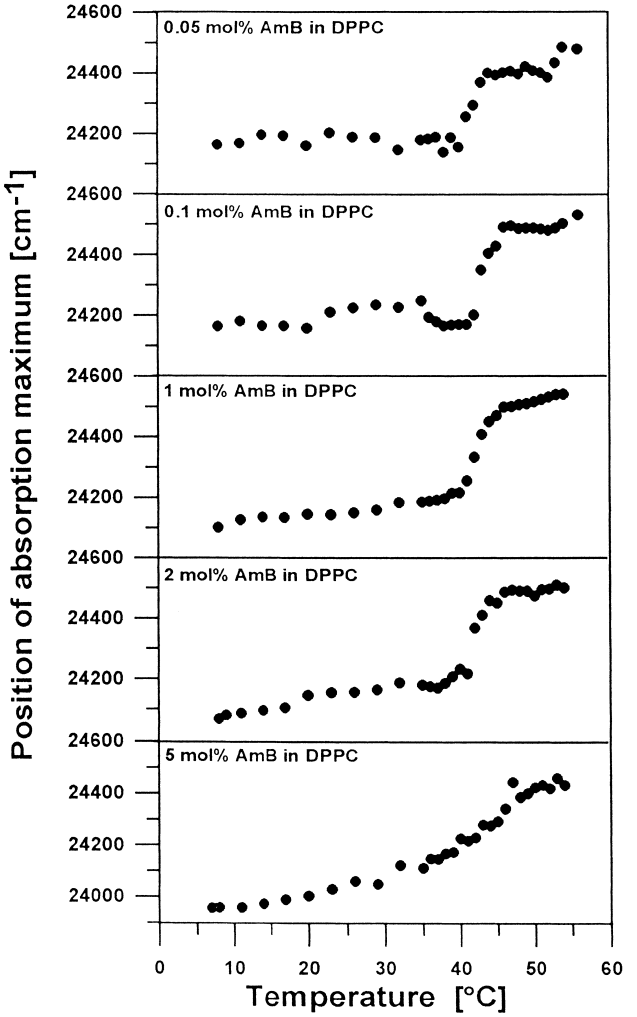

Fig. 4. Temperature dependences of the position of the 0-0 absorption maximum (in wavenumbers) of monomeric AmB incorporated into DPPC liposomes at the various concentrations indicated.

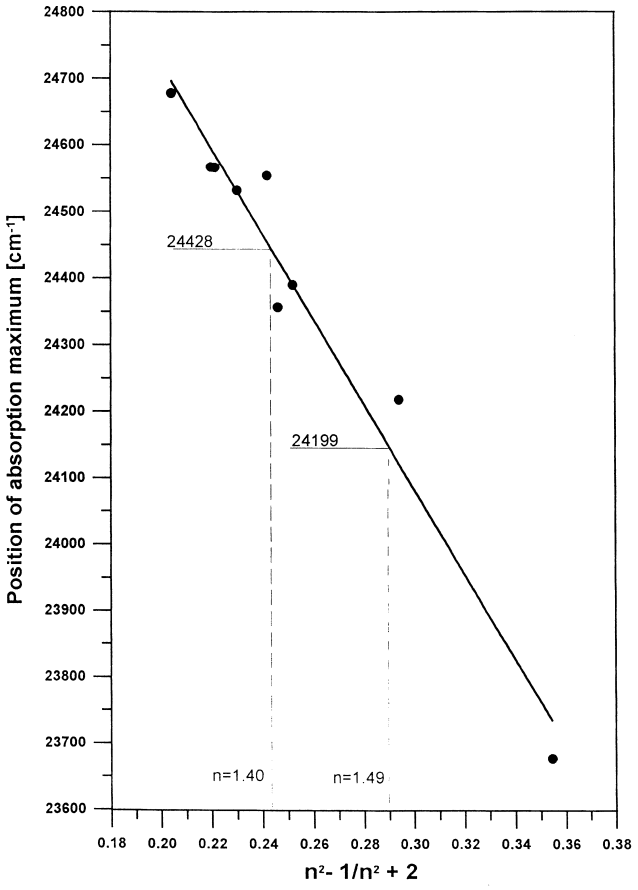

Fig. 5. Dependence of the position of the 0-0 absorption maximum of monomeric AmB recorded in different organic solvents on polarisability expressed by the refraction index *n*. The absorption maxima of AmB at 0.05 mol% with respect to DPPC, corresponding to 25°C and 50°C, are indicated along with the corresponding refractive index values following from the linear relationship.

phase is followed by a decrease in AmB aggregation level at 0.05 and 0.1 mol% of the drug. This effect is most probably directly related to the increase in the diffusional freedom of AmB within the hydrophobic core of the DPPC bilayer. Interestingly, the aggregation properties of AmB at higher concentrations are more complex in the phase transition temperature region. A decrease in the aggregation level is always preceded by a transient rise, following directly the phase transition temperature. According to numerous reports AmB broadens the phase transition thermograms; the onset of the transition is virtually the same as in the case of a pure lipid phase but, in addition, there are other components, observable at higher temperatures [8–11]. Termination of the transition was found to depend almost linearly on the AmB concentration in different lipid models up to 30 mol% of the drug [8,9]. Such behaviour expresses the heterogeneity of an AmB–lipid system. A rigid molecule of AmB interacts with the lipid alkyl chains by van der Waals forces [7] and acts like other similar structure-modifying agents, such as cholesterol [18] or membrane-spanning polar carotenoids [19]. Hydrophobic interactions of alkyl chains with AmB are most probably responsible for the formation of

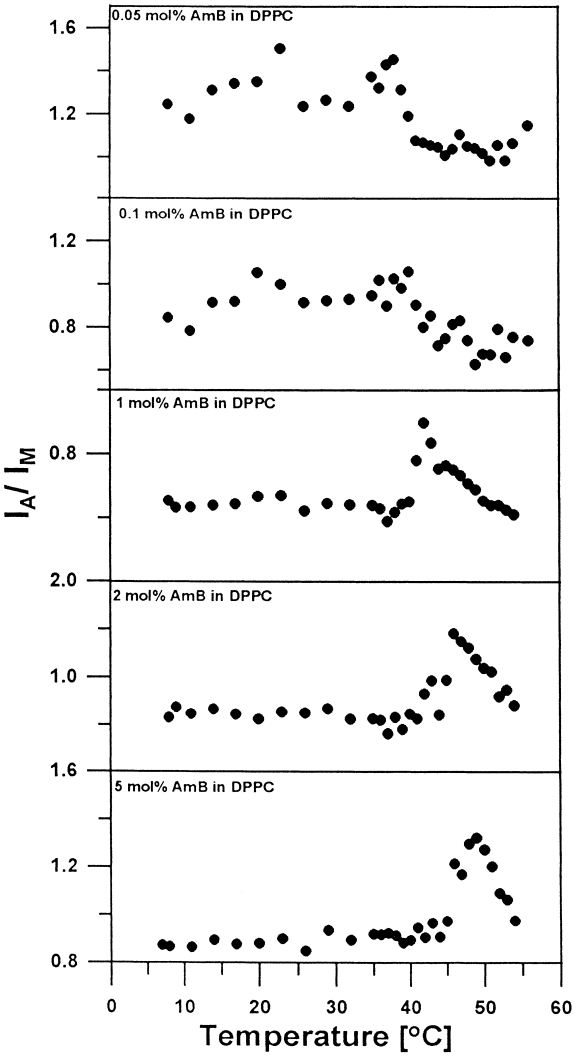

Fig. 6. Temperature dependences of the ratio of Gaussian components representing the aggregated structure I_A (centred at 334 nm) and 0-0 transition of the monomeric structure I_M produced on the basis of the Gaussian analysis of absorption spectra of DPPC liposomes containing AmB at the various concentrations indicated.

certain fractions of the lipid characterised by a phase transition temperature shifted toward higher energies. The two main subfractions have been characterised by the calorimetric technique in the AmB–DPPC system that passes through the phase transition at 42.6 and 46°C [11]. On the other hand, the linear relationship of the termination of the phase transition with AmB concentration reflects the coexistence of a higher number of different cooperative units in the lipid phase. As may be seen from Fig. 6, the threshold temperature, at the beginning of which the AmB aggregation level decreases in response to the rise in the incubation temperature, depends on the concentration of AmB in the lipid phase. This dependence is displayed in Fig. 7. The threshold temperature most probably corresponds to the transition to the fluid phase of DPPC. The enhanced aggregation of the drug in the phase transition region seems to be a direct effect of the attenuation of the AmB–lipid interactions owing to the mismatch in the thickness of the hydrophobic core of the DPPC membrane (32.9 Å [20]). The thickness of the hydrophobic core of the DPPC membrane in its gel state (39.4 Å [20]) is almost exactly double the hydrophobic segment of the AmB molecule [3] (see also the discussion in [11]). The temperature-induced deaggregation of AmB in the fluid phase of the membrane seems to be based on the same, purely thermal, effects as in the case of AmB molecular assemblies in an aqueous solution [21]. Fig. 8 presents an example of an Arrhenius plot corresponding to this phase of the experiments and Fig. 9 presents the concentration dependence of the activation energy of AmB deaggregation calculated on the basis of the linear coefficient of the Arrhenius plots. Interestingly, the activation energy has a maximum value corresponding to 1 mol% AmB in DPPC. This is an indication that molecular aggregates of AmB formed at this particular concentration in the membrane are more stable than when they are formed under different conditions. On the other hand, the AmB aggregation level in the gel phase corresponding to this particular concentration is at a minimum (see Fig. 10). At very low AmB concentrations (less than 1 mol%) formation of molecular aggregates in the form of a regular cylinder constituted by six to nine molecules may be unfavourable and molecular ensembles such as dimers would be formed in the lipid phase [22,23]. Owing to the complex vibronic structure of the absorption spectrum of AmB the excitonic bands of dimeric structures would overlap the monomeric bands and the excitonic bands of other aggregated structures

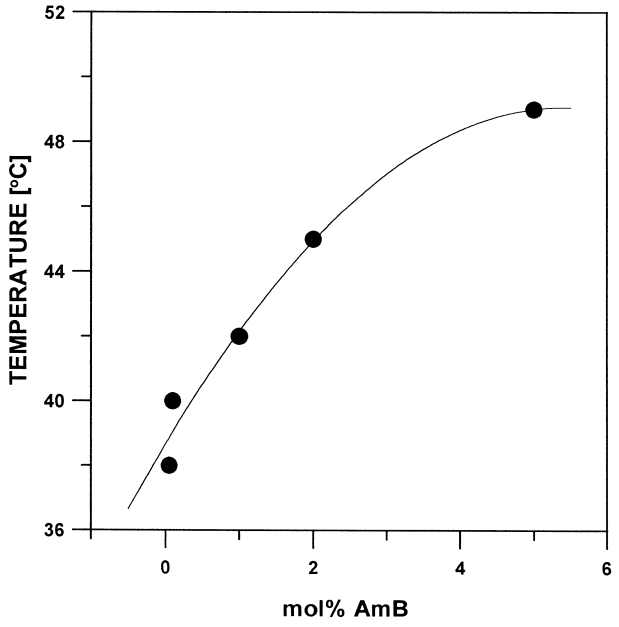

Fig. 7. Temperature of the termination of the main phase transition of DPPC membranes determined as an abrupt decrease in the aggregation level of AmB (see Fig. 6) as a function of concentration of AmB.

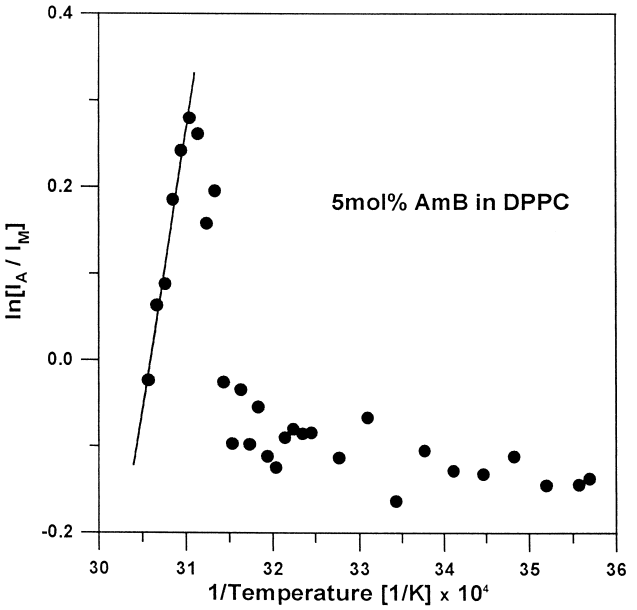

Fig. 8. Arrhenius plot of the I_A/I_M ratio (see Fig. 6) corresponding to DPPC liposomes containing 5 mol% AmB.

and therefore a precise description of all possible molecular forms based on this method would be difficult. The transition temperatures corresponding to the AmB concentrations below 1 mol% are below the phase transition temperature of the pure lipid phase (see Figs. 6 and 7). This is an indication that AmB acts as an 'impurity' with respect to the membrane, decreasing the order of the lipid matrix, at low molar concentrations. The fact that the aggregation level of AmB in the membrane in its ordered phase increases following an increase in concentration above 1 mol% (Fig. 10) most probably expresses the concentration-related increase in the probability of collisions of AmB within the lipid phase followed by the formation of molecular aggregates. The exciton splitting theory can be applied to the analysis of the formation of molecular oligomeric forms of AmB characterised by a hypsochromic spectral shift [14]. The spectral shift due to the formation of molecular aggregates can be expressed by the following equation [24]:

$$\nu(N) = \nu_{\mathrm{mon}} + 2\beta \cos\left(\frac{2m\pi}{N}\right) \quad (1)$$

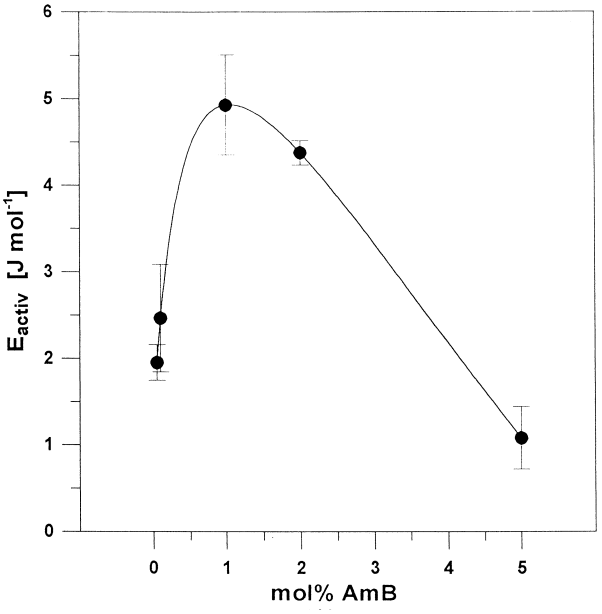

Fig. 9. Concentration profile of activation energy of temperature-related deaggregation of AmB in DPPC membranes determined on the basis of Arrhenius plots such as in Fig. 8. The points represent the mean of three independent experiments ± S.D.

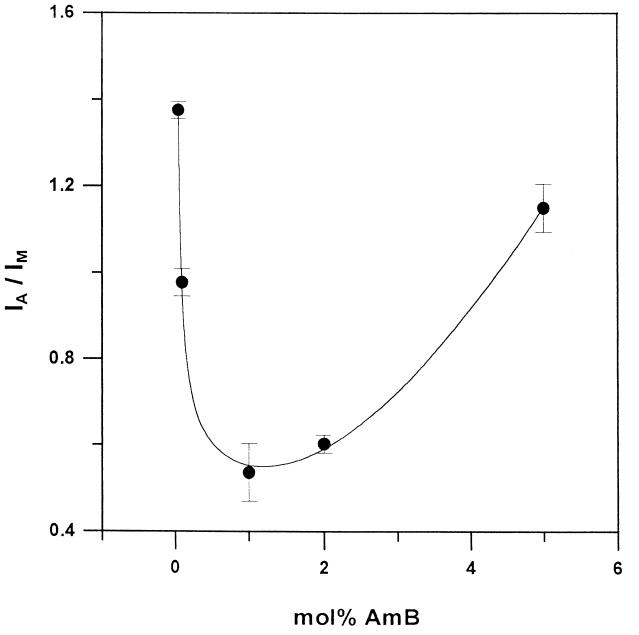

Fig. 10. Concentration profile of AmB aggregation level expressed as the I_A/I_M ratio (see Fig. 6), determined in the temperature region 5–35°C by fitting a line parallel to the ordinate axis to experimental points. The points represent the mean of three independent experiments ± S.D.

where m runs over all N exciton coupled states of the aggregate and β is the coupling matrix element expressed as:

$$\beta = \frac{|\mu_{\mathrm{mon}}|^2}{4\pi\varepsilon_0 n^2 R^3}(\cos\theta - 3\cos^2\phi) \quad (2)$$

In Eq. 2, ε_0 is the permittivity of free space, μ_{mon} is the transition moment of the monomer, n is the refractive index of the medium, R is the distance between nearest neighbours in the aggregate, θ is the angle between monomer transition moment vectors in the aggregate and ϕ is the angle between the transition dipole moment vector and the axis connecting the centres of transition moment vectors of neighbour molecules. One can substitute zero for parameter m in Eq. 1 in the case of the highest exciton state corresponding to the H-type aggregate. Fig. 11 presents a model of an AmB cylindrical aggregate formed with nine molecules. The molecular distances in such a model (chromophore distance R and pore radius r) can be calculated according to the experimentally observed spectral shifts, on the basis of the exciton splitting theory (Eqs. 1 and 2). The transition dipole of monomeric AmB was evaluated as 11.3

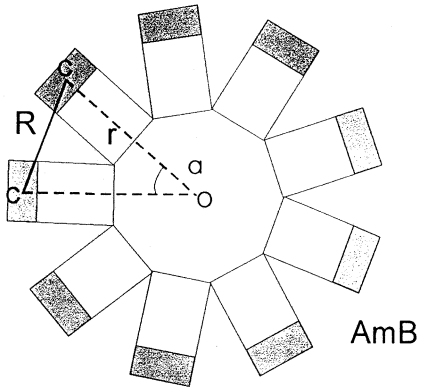

Fig. 11. Model of the aggregated molecular structure of AmB in the form of a hydrophilic pore. AmB chromophores are marked by the shaded area, R is the distance from the centre to the centre of the neighbour chromophore, r is the radius of the cylindrical structure.

Debye by integrating the absorption spectrum in 40% 2-propanol. The refractive indexes n of the DPPC membrane at 37 and 50°C were taken as 1.49 and 1.40 respectively, in accordance with the discussion above. The parameter θ is assumed to be 0 and parameter ϕ is assumed to be 90° in the case of a cylindrical structure of AmB. Considering the dimension of the AmB molecule ($d=6$ Å [14]) one can calculate the radius of a hydrophilic pore of a cylindrical aggregate as $r'=r-6$ Å. Pore diameters of 3.6 Å at 37°C and 4.2 Å at 50°C were calculated on the basis of the hypsochromic spectral shifts of AmB in DPPC membranes at 0.05 mol% concentration, on the assumption that cylindrical molecular structures of AmB were formed by nine molecules. A different number of molecules assumed to form a pore results in a slightly different pore diameter. For example, the same spectral shifts of AmB in DPPC membranes at 37 and 50°C are consistent with pore diameters of 4.3 and 5.3 Å in the case of a structure formed by 10 molecules and 1.2 and 2.0 Å in the case of a structure formed by eight molecules. According to the spectroscopic data analysed in terms of the exciton splitting theory it seems very probable that the hydrophilic pores of AmB in the DPPC hydrophobic phase are formed by nine molecules, owing to the fact that such structures are most probably stabilised not only by the van der Waals interactions among the chromophores but first of all by the hydrogen bonds among the polar AmB groups. The length of the hydrogen bonds seemed to correspond well with the diameter of the pore formed by nine AmB molecules according to the calculations presented above. On the other hand, the exact composition of an AmB pore in natural membranes may also depend on interactions with sterols that have been shown to be likely to bind to the AmB cylindrical structures. Interestingly, the spectroscopic data show that the diameter of the AmB channel increases upon transition from the lipid phase to the more fluid phase. This effect may be explained in terms of attenuation of hydrophobic AmB–lipid interactions, owing to a higher rate of *gauche-trans* isomerisation of alkyl lipid chains. According to several reports discussed in Section 1 the biological effect of AmB is higher with respect to membranes containing ergosterol compared to membranes containing cholesterol [1,2,21]. It is possible that the selectivity of AmB is directly related to the diameter of the pore-like molecular structure that is shown here to be dependent on the physical properties of the lipid matrix. Not only the different affinities of binding ergosterol and cholesterol to the AmB pores but also the well-known effect of sterols in decreasing the fluidity of lipid membranes (different in the case of ergosterol and cholesterol [11]) may be indirectly responsible for the selectivity of AmB with respect to the ergosterol-containing fungal membranes as compared to the mammalian membranes containing cholesterol. We plan to address this problem in our future research. Application of exciton splitting theory to the analysis of sterol- and AmB-containing lipid membranes may aid in evaluating the hydrophilic pore diameter formed in these different systems.

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